

DETERMINATION OF TROPANE ALKALOIDS IN HAIRY ROOT CULTURE OF *HYOSCYAMUS MUTICUS* USING DIFFERENT STRAINS OF *AGROBACTERIUM RHIZOGENES*

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ABSTRACT

Hairy Root Culture is the new route for large scale secondary metabolite production because of their fast and plagiotropic growth, genetic and biochemical stability. In this study, *Hyoscyamus muticus* hairy root clones were established following infection with *Agrobacterium rhizogenes* strains A4, LBA 9402/12 and NRC 5149. The transformation frequency obtained with the LBA 9402/12 strain was found to be highest one (98.33%), then that of the A4 (76.00%), followed by NRC 5149 strain (31.33%). Subsequently, the culture condition for the hairy root were established, however, different hairy root lines showed different biomass at 4 weeks of culture under 16 h light/8 h dark photoperiod in liquid MS medium. The LBA 9402/12 hairy roots showed the longest log phase and prolonged for three weeks at stationary phase in comparison with A4 and NRC 5149. Significant differences appeared between the tropane alkaloid concentrations of the different hairy root clones and all explants of the intact plant. The maximum hyoscyamine and scopolamine concentration was found with Transformed roots with LBA 9402/12 (5.913 and 1.530 mg/gd.w.t), respectively.

KEYWORDS: *Agrobacterium rhizogenes*, Hairy Root, Tropane Alkaloids, Hyoscyamine, Scopolamine and *Hyoscyamus muticus*

INTRODUCTION

Tropane alkaloids, especially hyoscyamine and scopolamine, are widely used in medicine for their mydriatic, antispasmodic, anticholinergic, analgesic and sedative properties (Zehra et al, 1999). Currently, these alkaloids are industrially extracted from various solanaceous plants belonging to the genera *Atropa*, *Duboisia*, *Datura* and *Hyoscyamus* (Griffin and GD, 2000, and Holzman, 1998). Tropane alkaloids are mostly synthesized in roots and then transported to the aerial parts of the plant (Oksman-Caldentey, 1987). Different species of *Hyoscyamus* are known to be rich sources of tropane alkaloids (Oksman-Caldentey, 1987), and amongst all of them the alkaloid content in *H. muticus* (Egyptian henbane) was found to be the highest (Zolala et al, 2007). Egyptian henbane is a member of the Solanaceae family, which is one of the large drug producing families (Mahran, 1967). Much research has, therefore, been conducted on this plant to discover a suitable alternative method for accelerating production of tropane alkaloids through *in vitro* procedures, including callus and suspension cultures, protoplast cultures, somatic hybridization and root cultures (Koul et al, 1986, Oksman-Caldentey et al, 1987, Oksman-Caldentey, K. M, Strauss, A. and Hiltunen (1987), Oksman-Caldentey, 1987, Oksman-Caldentey, 1986 and Oksman-Caldentey and Strauss 1986).

Transformed hairyroots were generated in some tropane alkaloidproducing plants through transformation by *Agrobacterium rhizogenes*; the possibility of alkaloid production in cultures of these transformed roots was studied by Knopp *et al.* (1988). *A. rhizogenes* able to transfer a part of its DNA (T-DNA), carried on a large plasmid (Ri plasmid), to the genome of the host plant (Limamiet *al.* 1998). Integration and expression of T-DNA genes in the host plant cells leads to the development of hairy roots which can be excised and grown *in vitro* as hairy root cultures. The hairy roots have received considerable attention from many researchers for the production of plant secondary metabolites e.g. cadaverine and anabasine in hairy root of *N. tabacum* (Fecker *et al.* 1993), anthraquinone and alizarin in hairy roots of *Rubiaperegrina* L. (Lodhi *et al.* 1996), and Scopolamine and hyoscyamine in *Daturainnoxia* (Dechaux and Boitel-Conti 2005) due to the large biomass production within a short period. Also they can be grown on a hormone free medium (Shanks and Morgan, 1999). These fast growing hairy roots are genetically stable and possess the whole biosynthetic potential of wild type roots (Hu and Du, 2006). Moreover, transformed roots are able to regenerate whole viable plants and maintain their genetic stability during further sub-culturing and plant regeneration (Giri and Narasu, 2000, Oksman-Caldentey *et al.* 1991, Sevon, *et al.* 1997, and Sevon *et al.* 1995). The present study is aiming to investigate the influence of various strains of *Agrobacterium* on the hairy roots development and quantify the range of alkaloids produced in hairy roots in comparison with different explants i.e. leaves, stems, and roots of the non-transformed plants.

MATERIALS AND METHODS

Hairy Root Development: Three strains of *A. rhizogenes* were used i.e. NRC 5149 (obtained from Agriculture Microbiology Department, National Research Centre), A4 and LBA 9402/12 (VTT Bio and Chemical processes, Plant biotechnology group). Prior to inoculation each bacteria strain was cultured in liquid LB medium (Luria Bertani media) (Lennox, 1955) at 28°C on an orbital shaker at 100 rpm to rich OD= 0.6 in 600 nm.

Seeds of *H. muticus* were surface sterilized as described by (Pandey and Chand, 2005) and cultured on a solid MS medium (Murashige and Skoog (1962) with 0.8% agar for germination and seedling development for 8 weeks. Leave segments were cut into almost 2 cm² and dipping for 20 min with *A. rhizogenes* strains suspension plus 500 µM Acetosyringon. Explants were blotted dry on sterile filter-paper to remove excess bacteria and placed on solidified MS medium at 28°C in the dark for two days. Explants were sub-cultured onto fresh media containing 500 mg/l Cefotaxime at 25±2°C 8/16 hour photoperiod for 3 weeks to eliminate bacteria from the outer surface of the plant tissue.

PCR Analysis for *A. rhizogenes* T-DNA: For polymerase chain reaction (PCR) analysis DNA was extracted from non-transformed roots as a negative control, bacteria strains as a positive control and from initiated hairy roots. Total DNA were isolated from the three *A. rhizogenes* strains (A4, 9402/12 and NRC 5149) using an Invitrogen Kit (Cat. No. CS11301). DNA was isolated also from hairy roots and plant roots according to Dellaporta *et al.* (1983). For the PCR reactions, specific primer with 5' end sequence "GGA ATT AGC CGG ACT AAA CG" and 3' end sequence "CCG GCG TGG AAA TGA ATC G" as described by Hazaa *et al.* (2006) were used to amplify a 20 bp fragments of rol A gene. In the PCR amplification, the PCR mixture was prepared (2.5 µl 10x buffer, 2.5 mM MgCl₂, 2.5mM of each dNTPs) before 1 µl (20 ng) from each of the primer pairs and 0.2 unit TaqDNA polymerase (Alliance Bio Cat. No. M010TP20) were added to the mixture plus 2 µl from the DNA (20 ng) and d.d H₂O to a final volume of 25 µl. The thermal conditions of the reaction set as following; 94°C for 3 min for DNA denaturation, 30 repeated cycles of 94°C for 1 min, 55°C for 2 min for annealing, 72°C for 2 min for extension. The PCR products were analyzed using 0.8% agarose gel in 1x TAE buffer (Tris-Acetate-EDTA electrophoresis buffer), 40mM Tris, 20mM acetic acid, 1mM EDTA) and stained with ethidium bromide (10 µl/ml)

for 10 min (Sambrook et al, 1989). Bands were visualized by examination under a UV trans-illuminator and photographed using a FUJIFILM DIGITAL CAMERA Fine Pix S9100/ Fine Pix S9600.

Growth Pattern of Hairy Root: For growth pattern study, 150 mg initiated hairy root fresh weight were cut and transferred to 20 ml fresh liquid MS medium and incubated continuously on an orbital shaker at 100 rpm and 25°C, under 16 h light and 8 h dark. For 6 weeks. Each week three flasks were harvested for the analysis of fresh weight. The results were expressed in percentage transformation frequency.

$$TF = \frac{\text{Number of pricks showing hairy root emergence}}{\text{Total no. of pricks}} \times 100$$

Alkaloid Analysis using the HPLC: Fresh weight samples (400 mg) from the hairy roots and normal explants (leaf, stem and root) were collected and freeze dried for 24 hours before grinded into powder. 10 mg powdered sample was weighed and subjected to the extraction of alkaloids (Hyoscyamine and scopolamine) using 10 ml extraction solvent mix of CHCl_3 : MeOH : NH_4OH (15:5:1 v:v:v), sonicated for 10 min, then kept at room temperature for 1 hour as described by Kagei et al. (1978). After filtration, the residues were washed twice with 1 ml of CHCl_3 . The pooled filtrate was evaporated to dryness and 5 ml of CHCl_3 and 2 ml of 1N H_2SO_4 were added to the residues and mixed well. The CHCl_3 phase was removed and the H_2SO_4 phase was adjusted to pH 10 with 28% NH_4OH in an ice-bath. From the solution, alkaloids were extracted twice, first with 2 ml and second with 1 ml of CHCl_3 . The combined extracts were filtered using 0.2 μm PTFE membrane filter after adding anhydrous Na_2SO_4 and then the residues were washed with 1 ml of CHCl_3 . The combined filtrates were evaporated to dryness at 40°C and sample residues were dissolved in 1 ml methanol alcohol for HPLC analysis. Hyoscyamine and scopolamine were analyzed in the HPLC column (Phenomenex Luna 5u C18 column) with dimensions of 250 x 4.6 mm and SPD – 10 A UV detector adjusted at λ_{max} of 235 nm. The mobile phase was acetonitrile: deionized water (65: 35) with flow rate of 2 ml/min. (Boitel Contiet et al. 2000).

RESULTS AND DISCUSSIONS

Different growth conditions were experimented for the purpose of various combinations which resulted in the finding of the most suitable growth condition for the hairy root, which was dipping the explants for 20 min in bacterial suspension and incubated for 2 days under dark on MS medium before subcultured to MS medium containing 500 mg/l of cefotaxime for 16 days under 16/8 h (day/night). Wounded *H. muticus* leaves were highly responsive to inoculation by each strain of the *A. rhizogenes*, as shown by the percentage of leaf explants from which hairy roots emerged (Table 1).

All the strains of *A. rhizogenes* used in this study were able to produce hairy roots at the site of infection of explants (Figure 1). It was found that there was no difference in the morphological characteristics between hairy roots induced by the three strains of *A. rhizogenes* (Figure 2).

Table 1: Effect of Different Strains of *A. rhizogenes* on the Frequency of Infection and the Growth of *H. muticus* Hairy Root

Bacterial Strains	Transformation Frequency %
A4	76.00 ±0.47
LBA 9402/12	98.33 ±0.98
NRC 5149	31.33 ±0.72



Figure 1: Hairy Root Initiation on solidify MS Medium from *Hyoscyamus muticus*



Figure 2: Morphological Characteristics for Hairy Roots Induced by the Three Strains of *A. rhizogenesi*. e. A) A4, B) LBA 9402/12 and C) NRC 5149

The transformation efficiency of LBA9402/12 was more efficient than that of A4 and NRC 5149. In *H. muticus* hairy root clones, LBA 9402/12 produced the highest transformation frequency with 98.33% followed by A4 (76%) and the lowest infection frequency (31.33%) was displayed in the strain NRC 5149. The superiority of LBA 9402/12 over A4 strain was corroborated with earlier investigations on several plant species including *Solanum dulcamara* (Chand, 1988), *Picrorhiza kurroa* (Verma *et al*, 2007) and *Amaranthus tricolor* L. (Swain *et al*, 2010). However, this is not in consonance with the results obtained with *Valeriana wallichii* (Banerjee *et al*, 1998), *Hyoscyamus albus* and *H. muticus* (Zehra *et al*, 1999) and *Coleus forskolii* (Garg, 2001), where the reverse held good.

PCR analysis resulted in successful amplification of a *rolA* products in all the three hairy root clones analyzed (Figure 3).

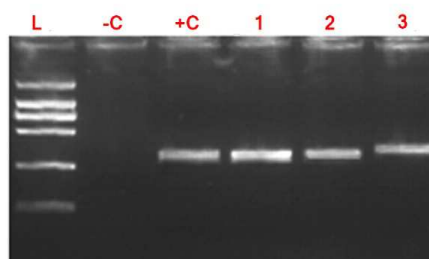


Figure 3: Lane L, 1Kb Ladder DNA, -C: Non Transgenic Root, +C: Bacteria (*Rol A*), 1: Hairy Root (A4), 2: Hairy Root (9402/12), 3: Hairy Root (NRC 5149)

The PCR products were absent in reactions performed with DNA isolated from normal plant roots (-C). Thus, it has been confirmed at molecular level that the T-DNA of Ri plasmid had been expressing into the genome of the Egyptian Henbane. In 2007 (Tiwari *et al.*), they confirmed the integration and expression of Ri T-DNA genes in dry root samples from 4 different strains of *A. rhizogenes* and its ability of causing genetic transformation of *Gentiana macrophylla* (Tiwari *et al*, 2007).

Growth Pattern of Hairy Root: Hairy roots were excised from the necrotic explant tissues and subcultured on fresh liquid MS medium without a supplemented hormones under light conditions for 6 weeks. The three strains of *Agrobacterium rhizogenes* (LBA 9402/12, A4 and NRC 5149) showed significant differences on the growth process in hairy root cultures of *H. muticus* (Figure 4). The growth kinetics of the hairy root and mean doubling time were calculated suspension culture flasks by linear regression of the plot of natural logarithms of the root biomass versus time from the original inoculum fresh weight level (150 mg). During the first week, a lag phase was observed in all hairy root clones. After that the biomass grew rapidly up to 2 weeks of the culture with different growth rates of each clone. The LBA 9402/12 showed the longest log phase which continued from 2 weeks in comparison with A4 and NRC 5149 which only limited for one week. Later, it was observed a slow growth phase (stationary phase), which showed that LBA 9402/12 clones was prolonged for three weeks at stationary phase while clones A4 and NRC 5149 were continued only for two weeks. Similarly, a different category for growth was reported among 4 different strains of *A. rhizogenes* (A4GUS, R 1000, LBA 9402 and ATCC 11325) by Tiwari *et al*, (2007).

From these results, it was indicated that LBA 9402/12 clone have a longest growth curves as compared with the other two clones A4 and NRC 5149 of *Hyoscyamus. muticus*.

Determination of Tropan Alkaloids: The Three different *A. rhizogenes* strains: A4, LBA 9402/12 and NRC 5149 were investigated to check their ability of transformation and production of tropan alkaloids *in vitro* from *H. muticus*.

In order to investigate that; HPLC method was applied to determine both main tropan alkaloids, i.e. scopolamine and hyoscyamine concentration in non-transformed root, stems and leaf samples, callus induced from plant leaf as well as the three clones of genetically modified root samples of *H. muticus* as illustrated in Figure 5 and Table 2.

All analyzed samples were particularly rich in hyoscyamine over scopolamine. Transformed hairy root clone LBA 9402/12 and A4 showed the highest concentrations of hyoscyamin (5.913 and 4.413 mg/gd.w.t) respectively, to increase between almost 4 to 5 fold than with NRC 5149. In case of scopolamine the concentrations reached (1.53 and 0.65 mg/gd.w.t) respectively, which give 3 to 7 fold above NRC 5149. The concentrations of hyoscyamin in root clone LBA 9402/12 is increased by 2.3, 7.2 and 3.6 folds to untransformed leaf, stem and root, respectively. Meanwhile scopolamine concentration increased by 4.9, 30.4 and 12.6 folds to untransformed leaf, stem and root, respectively.

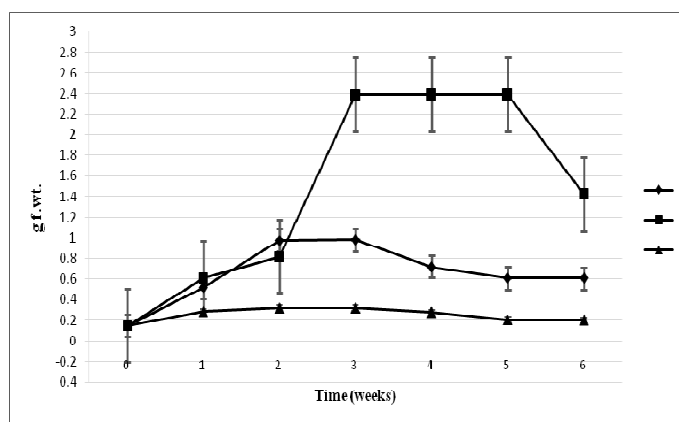


Figure 4: The Biomass Growth Pattern (g f.wt.) of the Three Hairy Root Lines (A4, LBA 9402/12 and NRC 5149) of *H. muticus* Cultured on Free – Hormons MS Medium for different Time Durations. Error Bars Indicate SE

Among the three agrobacteria strains, the hyoscyamine and scopolamine concentrations were higher in the clones LBA 9402/12 than A4, whereas in the NRC 5149, it remained very similar to untransformed plant parts. It is well known that the biosynthesis of tropane alkaloids takes place in the roots and that they are transferred into the leaves and preferentially stored as scopolamine (Hashimoto *et al*, 1991).

Previously, other studies have also reported the differential efficiency of various *A. rhizogenes* strains in promoting not only the induction and growth but also the secondary metabolite production of hairy roots. For example, different *A. rhizogenes* strains affected growth rate, saponin production and the ratio of different astragalosides in transgenic root cultures of *Astragalusmongholicus*Bge (Ionkova *et al*, 1997). The strain of *Agrobacterium* also influenced the development, growth rate and tropane alkaloids production in transformed root cultures of *H. muticus* (Vanhala *et al*, 1995 and Mateuset *et al*, 2000).

Hairy root cultures of *Gentianamacrophylla* were established by infecting leaf explants with four *A. rhizogenes* strains and each hairy root line showed different response regarding growth and production of secoiridoidglucosidegentiopicroside in transformed hairy root cultures (Tiwari *et al*, 2007). Clearly, the selection of an effective *Agrobacterium* strain for the production of transformed root cultures is highly dependent on the plant species, and must be determined empirically.

Table 2: Scopolamine and Hyoscyamine Concentrations (mg/gd. w. t) in Hairy Root Clones, Callus and different Parts of *H. muticus* plant

Analyzed Samples	Scopolamine (mg/gd.w.t)	Hyoscyamine (mg/gd.w.t)
Leaves	0.316±0.011	2.506±0.031
Stems	0.046±0.002	0.816±0.011
Roots	0.126±0.005	1.616±0.009
Transformed roots with A4	0.650±0.004	4.413±0.011
Transformed roots with LBA 9402/12	1.530±0.008	5.913±0.032
Transformed roots with NRC 5149	0.206±0.005	1.160±0.009

Data Represents Mean Values ± SE of Three Replicates

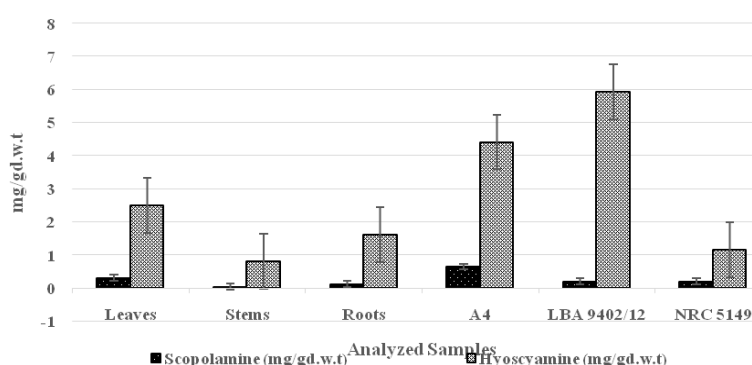


Figure 5: Scopolamine and Hyoscyamine concentrations in Hairy Root Clones, Callus and different Parts of *H. muticus* Plants. Bars Represent S. E

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